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Killing of bacteria by copper, cadmium, and silver surfaces reveals relevant physicochemical parameters

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The killing of bacteria on metallic copper surfaces in minutes to hours is referred to as contact killing. Why copper possesses such strong antimicrobial activity has remained enigmatic. Based on the physicochemical properties of metals, it was recently predicted that cadmium should also be active in contact killing [Hans *et al.*, Biointerphases **11**, 018902 (2010)]. Here, the authors show that cadmium is indeed antimicrobial. It kills three logs of bacteria in 9 h, compared to copper which kills eight logs of bacteria. Metallic silver kills less than one log of bacteria in 9 h. These findings support the novel concept whereby oxide formation, metal ion dissolution, and a Pearson soft character are the key factors for a metal to be antibacterial. Based on these parameters, copper and cadmium are expected to be the two most antibacterial metals. © *2017 American Vacuum Society*. [http://dx.doi.org/10.1116/1.4980127]

I. INTRODUCTION

Bacteria are rapidly killed on surfaces of copper or copper alloys containing at least 60% copper.[1] This process, also called "contact killing," is now well established and has explicitly been shown for many species of bacteria, yeasts, and viruses.[2] Because of their antimicrobial properties, copper and copper alloys lend themselves to the creation of self-sanitizing surfaces. This has received great interest in the light of increasing nosocomial infections in Western hospitals. In a number of hospital trials, wards and intensive care units were fitted with copper alloy table tops, bedrails, door handles, light switches, bathroom fixtures, or copper-impregnated linens and surfaces, in an effort to curb nosocomial infections.[3–5] The available data show a substantially reduced bacterial burden on critical surfaces and a reduced nosocomial infection rate in "copperized" hospital wards.[6,7] However, further data are needed to rigorously demonstrate that the use of copper leads to a lasting reduction of nosocomial infections.

The killing of bacteria by copper is not only of interest from a practical point of view but also raises two questions of fundamental interest: first, how are bacteria killed in contact killing, and second, what are the special properties of copper that make it antimicrobial? Substantial work has been performed to address the first question, and a concept of the killing mechanism has emerged. One key element involved in contact killing is the release of copper ions from the metal surface. These cause severe damage to the bacterial envelope, accompanied by the loss of membrane potential and cytoplasmic solutes, massive influx of copper ions into the cell interior, oxidative damage to cell constituents, and DNA degradation.[8–10] The importance and the order of the different processes leading to cell death may depend on the type of microorganism.[11] Bacteria–metal contacts also appear to be important for contact killing, but the mechanistic aspect of the process remains unexplained.[12]

All bacteria possess multiple systems to deal with toxic metal ions. Some of these are nonspecific, like cytoplasmic glutathione and metallothioneins, which bind a range of heavy metals. Others are specific for copper and are part of the copper homeostatic mechanism present in all bacteria, a key element of which is the copper ATPase that expels excess copper from the cytoplasm.[13] In addition, plasmid-encoded systems can provide tolerance to heavy metal ions by encoding heavy metal ion pumps or binding proteins.[14,15] However, bacterial resistance and homeostasis mechanisms only have a marginal effect on contact killing. Over nine logs of *Escherichia coli* bearing the plasmid-borne *pco* copper resistance system were killed on copper in 10 min, compared to 8 min for the wild-type.[8] Similarly, seven logs of copper tolerant *Salmonella* were killed in 15 min, compared to 10 min for the wild-type.[16] As expected, bacterial knockout mutants in copper resistance genes become more susceptible to contact killing by copper, but again the effect is maximally twofold.[9,10] Through these and other findings, it has also become clear that there are fundamental differences between bacterial heavy metal ion toxicities in culture and in a contact killing setting.

The second question regarding contact killing by copper has remained enigmatic: what are the special properties of

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copper that make this metal so antimicrobial and sets it apart from other metals? Hans *et al*. recently addressed this question in a meta-analysis comparing the physico-chemical properties of copper and copper oxides with those of other metals and their oxides.[17] They proposed that the key properties required for efficient contact killing are (1) the oxidation of the metal under ambient conditions (e.g., in air of moderate humidity), (2) high solubility of the ensuing metal oxides, and (3) a soft ionic character by the hard-soft acid-base (HSAB) Pearson concept (high thiophilicity) of the dissolved metal ions.[43] Conditions (1) and (2) lead to a high rate of ion release from the solid metal surface, while condition (3) is associated with the toxicity of metal ions to bacteria.[18] This concept led to the prediction that cadmium should also be effective in contact killing of bacteria, while metallic silver should be rather inert. We here show that these predictions are met: cadmium exhibits contact killing of *E. coli* at about one-third of the rate of copper, while silver is essentially inactive in the process. For the sake of direct comparison, contact killing by copper was also measured under the conditions used here.

The fact that cadmium ions are toxic to bacteria has been known for a long time. Glutathione and metallothioneins which protect cells from copper toxicity also provide protection against cadmium toxicity at low exposure levels.[13] Plasmid-borne Cd resistance systems can provide additional protection against cadmium toxicity. Molecular characterization of such Cd resistance systems in fact led to the identification of the first heavy metal ATPase. It is a P1B-type ATPase like the copper ATPases and can expel cadmium ions across the membrane.[19] Cadmium ions are also toxic and carcinogenic to humans.[20,21] Today, government regulations in western countries severely limit the use of cadmium, and it has disappeared from most common consumer products like paints or plastics.[22] However, Cd it is still used in batteries, electroplating in the aviation industry, and other specialty applications. In the light of its toxicity, Cd cannot be employed as antibacterial metal or in antibacterial coatings which come into contact with humans. But cadmium may serve as an alternative experimental model system to gain insight into contact killing mechanisms.

II. MATERIALS AND METHODS

A. Bacterial strains and growth conditions

E. coli K12 was grown aerobically overnight in 5 ml-cultures of Luria-Bertani (LB) medium at 37 °C. The stationary cells were collected by centrifugation for 15 min at 5000 × g, resuspended in 5 ml of 0.1 M Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) *p*H 7, and centrifuged as before. Washing of the cells with HEPES buffer was repeated, and the cells were finally suspended in 5 ml of 0.1 M Na-HEPES *p*H 7. The cells were kept on ice and immediately used for contact killing experiments. The cell concentration of these suspensions was calculated from the time 0 measurement on stainless steel. Cell concentrations remained constant within a few percent from experiment to experiment.

B. Preparation of coupons

Rolled metal sheets of 99.99% copper, 99.99% silver, 99.9% cadmium, and stainless steel AISI 304: X5CrNi18-10 were obtained from Wieland-Werke, Ulm, Germany. Coupons of stainless steel, silver, and copper of 1 × 2 cm were ground with silicon carbide abrasive paper of grid number P600 followed by cleaning in an ultrasonic bath with ethanol and air drying. A 600 cm^2 sheet of cadmium was cleaned manually with a Scotch pad and deionized water followed by washing with ethanol and air drying. The coupons and the cadmium sheet were used immediately after cleaning.

C. Measurement of contact killing

To assess contact killing, a wet plating technique was used, essentially as previously described.[9] Briefly, 20 μl of washed, resuspended cells were applied to coupons, forming a flat drop. Coupons with cells were incubated for 0–9 h in a water-saturated atmosphere at room temperature, which prevented any loss of volume in the drops. At the required time, the samples on the coupons were mixed by repeated pipetting to resuspend settled bacteria. A sample of 10 μl was then withdrawn and serially diluted in 0.9% NaCl followed by spreading on LB agar plates. After growth for 24 h at 37 °C, the colony forming units (cfu) were counted, and cell numbers were calculated relative to the originally applied 20 μl-samples.

D. Copper, cadmium, and silver determinations

For ion release measurements, the preparation of cells and coupons, incubation, and sample collection were identical to those used to measure killing, except that 10 μl of final samples withdrawn from the coupons were not used for cfu measurements but were diluted 300-fold with 1% HNO_3. Samples for cadmium measurements were diluted by an additional 100-fold with 1% HNO_3 to maintain the concentration in the valid calibration range of the measurement. Metal ion concentrations were determined on these samples by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx). Before the measurement, 1 μl of 10 mg/l scandium and cesium internal standard solutions were added per 1 ml of sample. Calibration was performed with standards of 0.1, 0.5, 2.5, 10, 50, 250, and 1000 mg/l of the respective element.

III. RESULTS AND DISCUSSION

A. Contact killing by copper

The antimicrobial properties of metallic copper are now well established and have been documented in numerous reports.[2] Widely different killing rates for bacteria on copper have been reported, ranging from seconds to hours. This variability is mainly due to wet versus dry plating methods and the buffers or media in which the bacteria were applied to copper and, to a minor extent, due to different surface roughness of the coupons and the bacterial strains investigated.[9,23–25] The frequently used phosphate-buffered saline contains a high chloride ion concentration which stabilizes Cu(I), which is considerably more toxic to bacteria than

Cu(II).[26] Tris buffer on the other hand strongly complexes Cu(II) and promotes its dissolution.[27] Applying cells to copper coupons directly in spent culture media also enhances the dissolution of copper due to the low *p*H of such media. For example, seven logs of wild-type *Enterococcus hirae* were killed in 12 min in Tris-Cl buffer, in 90 min in growth media, and in 6 h in water; in contrast, only about two logs were killed in 6 h in phosphate buffer.[9,23–25] On the other hand, differences in killing rates due to different bacterial strains are comparatively smaller: $>10^7$ cfu of five different bacterial biothreat agents were all killed in 0.5–5 min on copper in dry plating experiments.[28] Similarly, $>10^7$ cfu of five clinical isolates of methicillin-resistant bacteria were killed in 60–270 min, and in another study, >99% of ten clinical isolates were all killed in 2 h in wet plating on copper.[29,30] We here chose *E. coli* as the model organism because it has been used in many fundamental studies on contact killing. We also use neutral Na-HEPES buffer, which exhibits the least interference with the copper system.[31] However, this buffer system led to lower contact killing rates than those reported by us and others conducted under different conditions.

Published contact killing experiments have been conducted with a variety of bacterial strains and with varying cell preparations, buffers, and sample applications. We thus measured contact killing by copper, cadmium, and silver under identical conditions to allow direct comparison. Figure 1(a) shows the contact killing efficiency for copper coupons. Stainless steel was used as a reference, which does not show any significant reduction in cell survival in 9 h. Among the metals tested, the highest killing rate was observed for copper, which was included as a positive control. The starting number of 6.8×10^7 living cells declines essentially exponentially over the incubation time ($R^2 = 0.98$), resulting in no detectable live bacteria after 9 h. Contact killing is accompanied by the release of copper ions into the aqueous phase, as previously observed. Copper ion release is essentially linear over time ($R^2 = 0.98$), and copper accumulates in the aqueous phase at a rate of 0.13 mM/h to a final concentration of 1.26 mM after 9 h [Fig. 1(b)].

To understand the potential of copper in killing bacteria, the Pourbaix diagram is instructive [Fig. 1(c)]. It shows that under biosphere conditions [dotted parallelogram in Fig. 1(c): air, humidity, and organic substances], copper exists as metallic copper, Cu^{2+}, Cu_2O, and CuO. The thermodynamic view of these equilibrium states has to be supplemented by kinetic considerations. Presumably, ionic copper arises from the dissolution of Cu_2O and CuO. We previously showed that under wet plating conditions, there is a fairly even growth of CuO, while essentially no Cu_2O is detected after 5 h of incubation.[32] On the other hand, when copper oxidizes in air (tarnishing), Cu_2O is primarily formed. As shown previously, there is a correlation between oxide solubility (cf. Table I), copper ion release, and contact killing efficiency: Cu_2O is more active in contact killing than CuO and has a higher copper ion release rate, presumably due to the higher solubility of Cu_2O compared to CuO.[32] The high solubility of Cu_2O explains why tarnished copper retains its antimicrobial properties.

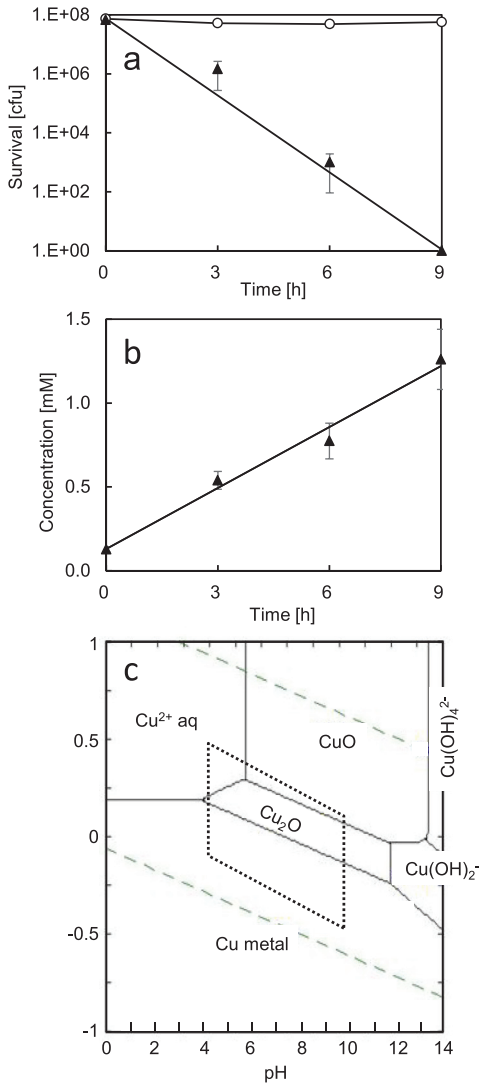

FIG. 1. Contact killing of *E. coli* on stainless steel and copper. (a) Cells suspended in Na-HEPES buffer were incubated on either stainless steel (○) or copper coupons (▲) at room temperature. At the times indicated, samples were withdrawn and survival was determined as detailed in Sec. II. (b) Copper coupons were incubated with cell suspensions as in (a). At the times indicated, samples were withdrawn and diluted with 1% (w/v) nitric acid, and the metal content was assessed by ICP-MS as described in Sec. II. The error bars in (a) and (b) indicate the standard deviations of three independent experiments. (c) Pourbaix diagram of copper, showing the speciation as a function of the reduction potential, E_h, and the *p*H. The dotted parallelogram delineates conditions encountered in the biosphere. The upper and lower diagonal dashed lines correspond to the reduction potentials of water saturated with oxygen and hydrogen, respectively, at 10 kPa. The diagram is based on published data (Ref. 40).

B. Contact killing by cadmium

On the basis of the theoretical considerations outlined above, cadmium surfaces should also be effective in contact killing, as Cd oxidizes under ambient conditions and the oxide is very soluble, and cadmium ions are soft (thiophilic) and thus toxic to bacteria.[17,18,33] Figure 2(a) shows that

TABLE I. Properties of selected metals ordered by oxide solubility.

Element/ion	Hard-soft property HSAB[a]	Oxide solubility $pK_{S[MeO/Me(OH)]}$[b]
Ag(I)	s	−7.7
Cu(I)	s	−9.0
Cd(II)	s	−13.6
Co(II)	i	−14.2
Ni(II)	i	−14.7
Fe(II)	i	−15.1
Pb(II)	i	−15.2
Zn(II)	i	−16.4
Cu(II)	i	−23.5
Hg(II)	s	−25.4
Sn(II)	h	−26.2
Al(III)	h	−32.9
Fe(III)	h	−37.4

[a]HSAB, Hard-soft acid-base character according to Pearson: h, hard; i, intermediate; s, soft (Ref. 43).
[b]pK_S-values of the metal oxide or metal hydroxide equilibria ($pK_{S[MeO/Me(OH)]}$) (Ref. 44).

contact killing on cadmium proceeds exponentially, as is apparent for copper, but at about one third of the rate observed for copper. Bacterial survival on cadmium is reduced by three logs over 9 h, compared to nearly eight logs/9 h on copper. However, the accumulation of cadmium in the aqueous phase is much more rapid than that of copper, proceeding at a rate of 9 mM/h in the first 3 h, and gradually slowing down to 2 mM/h at 9 h [Fig. 2(b)].

From the Pourbaix diagram for cadmium, it is apparent that metallic cadmium is not a stable metal under biosphere conditions [Fig. 2(c)]. In the presence of water, Cd^{2+} and $Cd(OH)_2$ are the prevailing forms of cadmium. $Cd(OH)_2$ has a solubility similar to CdO (cf. Table I), which is lower than that of Cu_2O or Ag_2O, but higher than that of CuO.[34] This does not explain the high dissolution of cadmium observed in the present experiment, but the solubility of CdO and $Cd(OH)_2$ is strongly affected by other ions in the system, and we suspect that the Na-HEPES buffer used in the present experiments augments cadmium solubility. Be it as it may, the observed contact killing by cadmium is in line with the expectations for a metal that oxidizes in the ambient and has a relatively high oxide (hydroxide) solubility and a soft HSAB character.

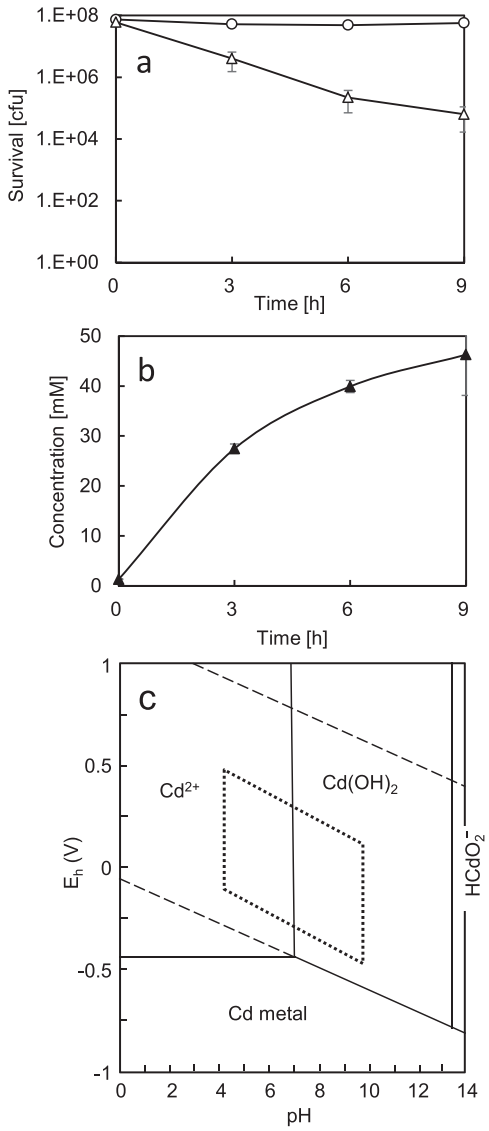

FIG. 2. Contact killing of *E. coli* on stainless steel and cadmium. (a) Cells suspended in Na-HEPES buffer were incubated on either stainless steel (○) or cadmium coupons (Δ) at room temperature. At the times indicated, samples were withdrawn, and survival was determined as detailed in Sec. II. (b) Cadmium coupons were incubated with cell suspensions as in (a). At the times indicated, samples were withdrawn, diluted with 1 wt. % nitric acid, and the metal content was assessed by ICP-MS as described in Sec. II. The error bars in (a) and (b) indicate the standard deviations of three independent experiments; note that some error bars are too small to be visible. (c) Pourbaix diagram of cadmium. See the legend of Fig. 1(c) for details. The diagram is based on published data (Ref. 41).

C. Contact killing by silver

Silver was included in this study because of its widespread use as an antimicrobial material. However, metallic silver which is not oxidized was shown already in 1937 to be devoid of antibacterial activity.[35] This was subsequently confirmed in various other studies, most recently by Rebelo *et al.*[36] In agreement with these findings, we found that silver does not exhibit detectable contact killing over 6 h and only marginal killing (less than 1 log) in 9 h [Fig. 3(a)]. Also, there is very little silver ion release into the aqueous phase, which proceeds linearly over time ($R^2 = 0.996$) at a rate of only 0.002 mM/h [Fig. 3(b)]. However, this still results in the release of 20 μM silver ions over 9 h. Why this does not cause significant killing may have several reasons. First, in the present experiments, the bacteria are nongrowing and may thus be less susceptible to toxic metal ions than growing cells. Second, even in culture, the inhibitory concentration of Ag^+ can vary widely, depending on the experimental conditions. Xu and Imlay showed that the concentration of Ag^+ required to inhibit the growth of *E. coli* (which is not equivalent to cell death) ranges from 0.1 to 20 μM, depending on the experimental conditions.[18] Clearly, inhibition of growth

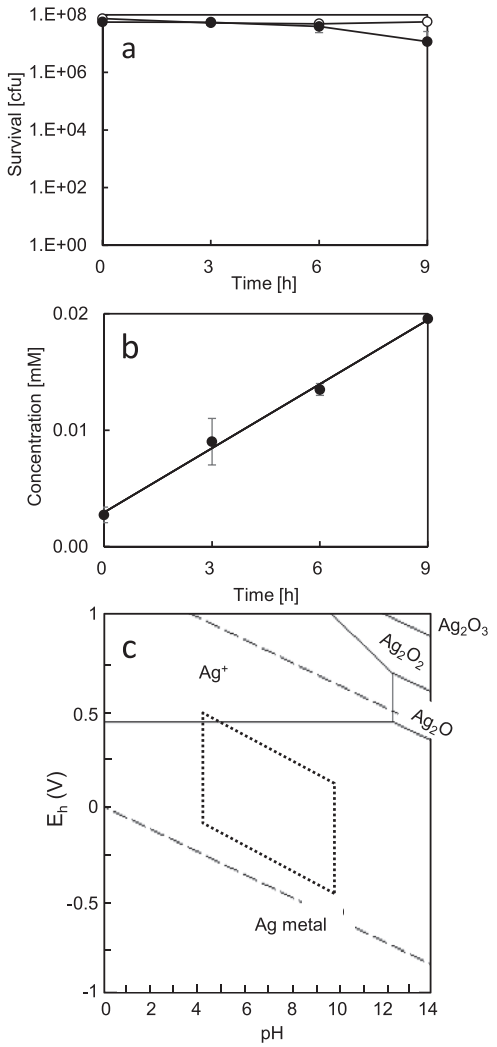

FIG. 3. Contact killing of *E. coli* on stainless steel and silver. (a) Cells suspended in Na-HEPES buffer were incubated on either stainless steel (○) or silver coupons (●) at room temperature. At the times indicated, samples were withdrawn, and survival was determined as detailed in Sec. II. (b) Silver coupons were incubated with cell suspensions as in (a). At the times indicated, samples were withdrawn and diluted with 1 wt. % nitric acid, and the metal content was assessed by ICP-MS as described in Sec. II. The error bars in (a) and (b) indicate the standard deviations of three independent experiments. (c) Pourbaix diagram of silver. See the legend of Fig. 1(a) for details. The diagram is based on published data (Ref. 42).

and the killing of nongrowing cells by heavy metal ions are different processes, but we are not aware of an investigation of the toxicity of Ag^+ to nongrowing bacteria.

The relative lack of contact killing by silver can be well explained by its physico chemical parameters and the Pourbaix diagram. Silver is essentially stable as a metal under biosphere conditions; oxides and Ag^+ ions only form at acidic *p*H and the most oxidizing biosphere conditions [Fig. 3(c)]. The numerous applications involving silver as an antimicrobial make use of silver ions or silver oxide, usually in the form of silver oxide nanoparticles.[37]

Kawakami *et al*. tested the antimicrobial activity of all the metals listed in Table I, except iron, cadmium, and mercury.[38] They found copper and silver to be the most efficient

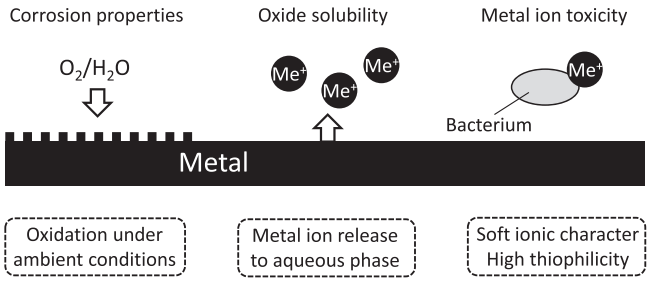

FIG. 4. Schematic of the key factors required for the antibacterial activity of a metal.

antimicrobial metals. However, it must be stressed that they used the Japanese testing protocol JIS Z2801. By this method, stationary bacteria in growth media are exposed to the surface to be tested for 24 h at 35 °C, conditions which favor oxidation and/or ion release. The JIS protocol is generally used for materials with an intrinsically low antibacterial activity, such as silver-doped plastics. When silver-doped refrigerator linings were tested by the JIS protocol, they killed one log of bacteria in 24 h at 35 °C but had no detectable antibacterial activity in 24 h at 5 °C.[39] Clearly, data obtained by the JIS protocol cannot be compared to the results of the wet plating technique used here and by others.

IV. CONCLUSIONS

This work supports the following requirements for contact killing, outlined in Fig. 4:

(1) Oxidation (corrosion) of the meal under ambient conditions, e.g., in air of moderate humidity.
(2) Release of substantial amounts of metal ions into the aqueous phase.
(3) Bactericidal activity of the metal ions due to their soft HSAB character/high thiophilicity.
(4) Based on (1)–(3), copper and cadmium are expected to be the two most antibacterial metals.
(5) These findings support the previous hypothesis of the physico-chemical parameters important for contact killing.[17]

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